

CANDIDATE LOCUS ANALYSIS FOR PHACE SYNDROME

by

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## ABSTRACT

Posterior fossa malformations, large infantile hemangioma of the head neck or face, arterial cerebrovascular anomalies, cardiac anomalies, and eye anomalies define the acronym for PHACE syndrome. PHACE syndrome (OMIM no. 606519) is a neurocutaneous syndrome with unknown etiology and pathogenesis. We report on an individual with PHACE syndrome with a complete deletion of *SLC35B4* in the 7q33 region. In order to further analyze this region, *SLC35B4* was sequenced for 33 individuals with PHACE syndrome and one parental set. Common polymorphisms with a possible haplotype but no disease causing mutation were identified. Sixteen of 33 samples of the PHACE syndrome patients were also analyzed for copy number variations using high resolution oligo-Comparative Genomic Hybridization (CGH) microarray. A second individual in this cohort had a 26.5kb deletion approximately 80kb upstream of *SLC35B4* with partial deletion of the *AKR1B1* gene in the 7q33 region. The deletions observed on 7q33 are not likely the singular cause of PHACE syndrome; however, it is possible that this region provides a genetic susceptibility to phenotypic expression with other confounding genetic or environmental factors.

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## INTRODUCTION

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Seven females with cervicofacial hemangiomas along with other anomalies were described by Pascual-Castroviejo in 1978 [Pascual-Castroviejo,1978]. This association was further defined, and the acronym PHACE was proposed in 1996 by Frieden *et al.* [Frieden et al.,1996]. PHACE syndrome includes **p**osterior fossa malformations, large infantile **h**emangioma of the head neck or face, **a**rterial cerebrovascular anomalies, cardiac anomalies, and eye anomalies. Sternal clefting and/or supraumbilical raphe can also be present, and is referred to as PHACES syndrome (OMIM # 606519) [Frieden et al.,1996] .

Large facial hemangiomas indicative of PHACE syndrome are often described as segmental; they comprise approximately 20% of hemangiomas identified in the head and neck region [Haggstrom et al.,2006]. In most cases, a diagnosis of PHACE syndrome includes a segmental hemangioma of > 5cm in diameter of the head, neck, or face and one extracutaneous anomaly. In 2009, leading experts established both major and minor criteria based on the particular organ system to better define PHACE syndrome [Metry et al.,2009a; Metry et al.,2009b]. Some of the major criteria include: anomalies of the major

cerebral arteries (dysplasia, arterial stenosis, and persistence of trigeminal artery), structural brain anomalies (Dandy-Walker complex, posterior fossa anomaly), cardiovascular anomalies (coarctation of the aorta, or aneurysm), ocular anomalies (posterior segment abnormality, retinal vascular or morning glory disc anomaly), and ventral or midline anomalies (sternal defects). Minor criteria include the persistence of embryonic arteries, midline anomalies, ventricular septal defects, cataracts, or hypopituitarism. A definition of PHACE syndrome based on these criteria would include a segmental hemangioma >5cm in diameter of the head, neck, or face plus either one of the major criteria or 2 minor criteria.

To date there have been more than 300 cases of PHACE syndrome reported in the literature [Haggstrom et al.,2010] but the etiology and pathogenesis of PHACE syndrome still remains unknown. To investigate a possible genetic origin for the pathogenesis of PHACE syndrome, genomic oligo-Comparative Genomic Hybridization (CGH) microarray was used to evaluate a patient with PHACE syndrome identifying a small novel deletion on 7q33. This region was subsequently analyzed in a larger cohort of PHACE syndrome cases and the results are reported here.

A small deletion of approximately 100kb in size located on chromosome 7q32 was identified in a patient that was diagnosed with PHACE. The affected gene in this region was SLC35B4. It is a glycosyltransferase that transports sugar from the cytoplasm to the Golgi apparatus [Ashikov et al.,2005]. This gene has not been defined in the literature as associated with any diseases.

## MATERIALS AND METHODS

This study was approved by the Institution Review Boards of the University of Utah, University of California San Francisco, Oregon Health & Science University and Children's Hospital of Wisconsin and informed consent was obtained from all participants.

### Subjects

DNA was obtained from 5 subjects with PHACE syndrome through the Utah Clinical Genetics Research Program, Istanbul Medical Faculty, Istanbul University, and DNA from 28 additional individuals with PHACE syndrome and 2 unaffected parents was obtained from the PHACE Syndrome International Clinical Registry and Genetic Repository. All affected subjects met diagnostic criteria for PHACE syndrome based on the diagnostic criteria of *Metry et al.* [Metry et al.,2009a]. Diagnostic criteria include a large hemangioma on the face measuring 5 cm or more plus at least one major criteria such as a posterior fossa anomaly, coarctation of the aorta, developmental anomalies of the great cerebral arteries, developmental posterior segment eye anomalies and midline ventral defects, such as sternal cleft. Information was obtained from physical examinations and medical histories (e.g., results from echocardiograms, brain and vessel imaging, and ophthalmologic examinations).

Participant 1 was born at 38 weeks gestation to a 21-year old primiparous woman



via Cesarean section due to abruptio placentae. A faint red birthmark involving the right side of the face in a segmental pattern was noted at birth. The infant was admitted to the neonatal intensive care unit for care. Magnetic resonance imaging (MRI) of the brain at 1 week of age showed a dysplastic right superior vermis, an absent inferior vermis, a hypoplastic right dural venous sinus, and an abnormal enhancement of the anterior chamber of the right eye. Repeat MRI imaging at 1 month of age showed an increase in the size and number of proliferating hemangiomas within the right frontal scalp, right orbit and soft tissues of the face. Additional intracranial foci were identified in the right petrous apex, subarachnoid space, and pituitary gland. The circle of Willis was noted to be aberrant and there was a slight increase in the amount of flow-voids in the supracerebellar cistern. Computed tomography (CT) of the chest and abdomen showed multiple contrast-enhancing lesions within the chest wall, right hepatic lobe, and bowel wall. Ophthalmologic exam showed sclerocornea, a persistent papillary membrane, and a small superior optic nerve. An echocardiogram was normal. There was no obvious sternal cleft. The infant developed necrotizing enterocolitis with bowel perforation requiring bowel resection. At surgery multiple hemangiomas were visible throughout the small intestines. A second surgery was required in which the entire small intestine was determined to be nonviable. Support was withdrawn at approximately 2 months of age in a series by Drolet *et al.* 2011 [Drolet BA,2011 (submitted)].

#### DNA Isolation

Genomic DNA was extracted from peripheral blood samples.

### Array CGH

A minimum of 500ng and up to 1µg of purified DNA was used on the microarray depending on the amount of sample available. Reference DNA that consisted of a pool from 10 healthy individuals was used as sex matched control (Promega GmbH, Mannheim, Germany) when the sex of the patient was known.

Further data analysis was performed for all of the samples using the Nexus Copy Number software (BioDiscovery, Inc., El Segundo, CA) to analyze compatible samples at one time. Deletions or duplications that were detected with 10 data points or more and had a log<sub>2</sub> ratio greater than  $\pm 0.3$  were considered significant. Quality scores of 0.200 or less were considered to be excellent. Scores between 0.200 and 0.300 were further evaluated by taking the total number of gains/losses (base pairs) and dividing it by the total amount of genomic material. Samples that had 0.5% or less variability were considered valid. The regions of deletions or duplications were compared between samples.

A total of 24 samples (22 proband, 2 unaffected parents) out of 35 had a sufficient amount of DNA to perform the human CGH 3 x 720K whole genome tiling array (Roche Nimblegen, Inc., Madison, WI) DNA labeling (Nimblegen Dual-Color DNA Labeling Kit, Roche Nimblegen, Inc., Madison, WI), hybridization (Nimblegen Hybridization Kit, Roche Nimblegen, Inc., Madison, WI), and washing were performed according to the package insert. Data analysis was performed using Nimblescan (Roche Nimblegen, Inc., Madison, WI) and results were viewed using SignalMap (Roche Nimblegen, Inc., Madison, WI). Further data analysis was performed for all of the samples using the Nexus Copy Number software (BioDiscovery, Inc., El Segundo, CA). Deletions or

duplications that were detected with 10 data points or more and had a  $\log_2$  ratio greater than  $\pm 0.3$  were considered significant. Quality scores that were calculated using the Nexus software were used to determine if the sample data were valid. Quality scores of 0.200 or less were considered to be excellent. Scores between 0.200 and 0.300 were further evaluated by taking the total number of gains/losses (base pairs) and dividing it by the total amount of genomic material. Samples that had 0.5% or less variability were considered valid. Any sample with a quality score that was greater than 0.3 was excluded. In addition, sample data exhibiting greater than 1% variability in the genome were excluded. The regions of deletions or duplications were compared between samples. For purposes of this report, the 7q33 region was specifically analyzed. Results from an additional archived 24 normal control samples were further analyzed for the 7q33 region exclusively. These archived normal control samples were processed using various CGH microarrays including 4 x 72K, 1 x 385K, and 1 x 2.1M microarrays.

### Sequencing

Whole genome amplification was performed on all of the samples for Sanger sequencing using the Illustra GenomiPhi DNA Amplification Kit (GE Healthcare Bio-Sciences Corp Piscataway, NJ). For cases in which there was not sufficient amount of DNA to perform the microarray (<500ng), only Sanger sequencing was performed on the selected candidate gene (*SLC35B4*).

Sanger sequencing using big dye terminator was performed on the *SLC35B4* gene 10 coding exons for 35 samples including 33 probands, and 1 unaffected parental set. Primers were designed to sequence exonic regions including exon-intron boundaries.

PCR was performed using approximately 50-75ng of genomic DNA. Forward and reverse primers were added at 10 $\mu$ M each with 12.5 $\mu$ L of High Fidelity PCR Master mix (Roche, Mannheim, Germany). Bidirectional sequencing was performed on a 3730 ABI DNA Genetic Analyzer, using forward and reverse primers. Analysis was performed using Mutation Surveyor (SoftGenetics, LLC., State College, PA) for any difference from the reference sequence that would result in a non-synonymous mutation. Single nucleotide polymorphisms that were identified were evaluated using Alamut (Interactive Biosoftware, France).

## RESULTS

All probands met diagnostic criteria for PHACE syndrome [Metry et al.,2009a]. Using the Nimblegen 3 x 720K whole genome tiling array, a novel small deletion (-0.7 log<sub>2</sub> ratio) of approximately 100kb in size located on chromosome 7q33 (chr7: 133,600,015- 133,700,204, NCBI36/hg18) was identified in participant 1 (see Figure 1A) (clinical description above). This is a small region containing one gene, *SLC35B4* (chr7: 133,624,630-133,652,343, NCBI36/hg18), that was deleted. Parental samples were not available in order to determine *de novo* or familial status of the deletion. This region is highly conserved (see Figure 1B).

Out of the 33 probands, in which sequencing was performed for one candidate gene (*SLC35B4*) based on the CGH microarray results, 11 probands showed no variation. In the other 22 probands 7 SNPs were identified in the introns and 1 synonymous SNP detected in the exon of *SLC35B4* (rs447266, rs34664116, rs2075372, rs1832052, rs1421484, rs2241336, rs2505, rs2504). All of these SNPs were previously identified and common within the population. In all but two cases, these SNPs (7 intronic, 1 exonic) were found together in each sample, indicating a possible haplotype. As expected, participant #1 with the *SLC35B4* deletion in this region did not have any SNPs in the heterozygous position. Subsequently, 24 samples (22-proband, 1 parental set-father and mother of participant #15) were run on the Nimblegen 3 x 720K whole genome tiling array, since there was an insufficient quantity of DNA for 11 of 35

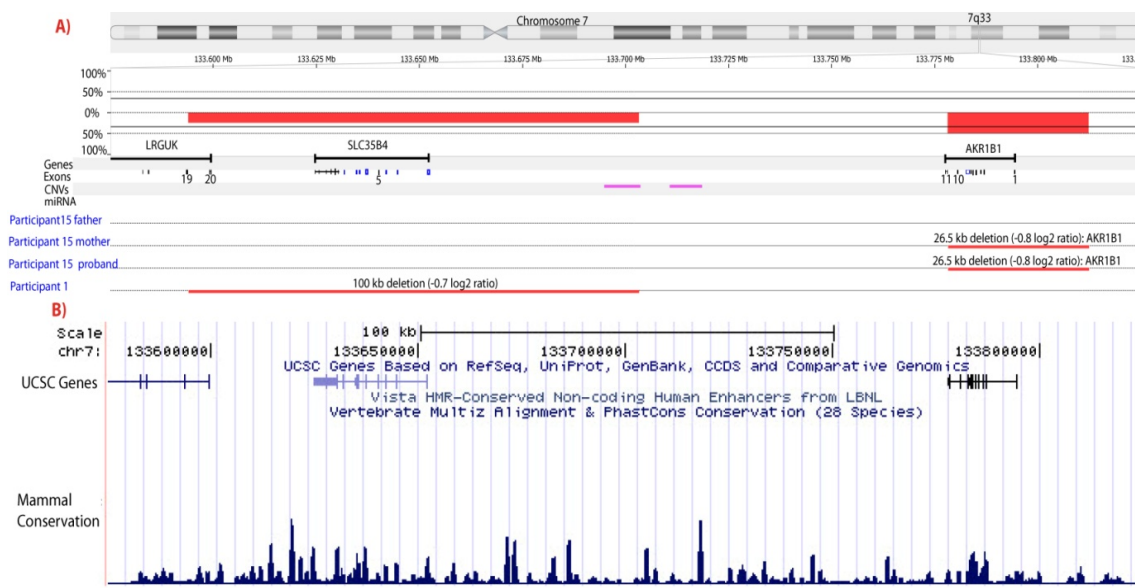


Figure 1A. Summary of affected probands plus the unaffected parental set. Figure 1B. Conservation of 28 mammalian species including 17 placental mammal species and human for the 7q33 region.

samples. Out of 2 samples run on the array, 6 samples were eliminated because either the quality scores were above 0.3 or due to aberrant microarray results. A total of 16 samples from the probands and unaffected parental set were CNVconsidered valid for analysis. CGH array data from 24 randomly selected healthy control samples were analyzed exclusively for *SLC35B4*. There were no deletions or duplications identified in this region for the healthy controls.

Interestingly, participant #15, had a 26.5kb deletion approximately 80kb upstream (chr7: 133,783,161- 133,809,611, NCBI36/hg18) of *SLC35B4* (clinical description in clinical report section). In this patient 16.3kb out of 16.8kb of the *AKR1B1* gene (aldo-keto reductase family 1, member B1) is deleted (*chr7:133,777,647-133,794,428*, NCBI36/hg18). Participant #15 is a Caucasian female born full term to a primiparous 27 year-old woman. The birth weight was 4kg. There were no reported pregnancy complications. The infant's hemangioma involved the right side of the face and scalp

with extension onto the neck and upper chest. A subglottic hemangioma was also present. The brain imaging was normal. An echocardiogram revealed an interrupted aortic arch and a ventricular septal defect. There were no eye anomalies or sternal defects.

Additional complications included scoliosis and unilateral hearing loss. The identical deletion was also identified in the mother. The mother did not have vascular anomalies, hemangiomas or other reported medical conditions. She had a normal echocardiogram, but an angiogram has not been performed. When the mother's CNV regions were compared with those of participant #15, nine novel deletion/duplication regions were identified in the child's sample. The percent variability in the genome was much higher for the child compared to the mother (0.13% versus 0.06%, respectively). The other 14 individuals with PHACE syndrome had no significant changes in the 7q33 region on the CGH microarray. There were no other non-CNV regions in the whole genome that contained genes where deletions or duplications showed an overlap in at least 2 samples. When one looked at CNV regions present in at least 25% of the samples from individuals with PHACE syndrome, seven CNV regions were identified. All of these CNV regions identified have been previously recognized as common CNVs in the normal population. Variability within the genome using our cut-off criteria for all samples ranged from 0.08% to 0.47% based on the total number of base pairs detected.

## DISCUSSION

Out of 16 individuals with PHACE syndrome in which CGH microarray results could be analyzed, 2 had small deletions on chromosome 7q33 region. This region contains *SLC35B4* and *AKR1B1*. *SLC35B4* (solute carrier family 35, member B4) is a glycosyltransferase that transports sugar from the cytoplasm to the Golgi apparatus [Ashikov et al.,2005]. No disease causing mutations are reported in the literature related with *SLC35B4* and *AKR1B1*. However, repeat dinucleotide and single nucleotide polymorphisms in the *AKR1B1* gene have been reported in association with diabetic macroangiopathy, diabetic retinopathy, and diabetic nephropathy, and cardiorenal complications [So et al.,2008; Thamotheampillai et al.,2006; Wolford et al.,2006; Xu et al.,2008]. None of them had deletions of *AKR1B1*. In addition, we showed that 24 healthy controls did not have any structural changes in this region and no CNVs have been reported in this deleted region. The deletions found in our 2 patients are novel changes not previously linked with any disease. The deletions in these 2 patients do not overlap; however, the breakpoints are 80 kb apart and located on the 5' upstream regions of *SLC35B4*. Regulatory proteins or transcription factors related to the regulation of *SLC35B4* binding sites may have been affected by these deletions in both patients. Expression studies have shown that *SLC35B4* is expressed in the fetal brain and heart tissue supporting it as a potential candidate gene [Pascual-Castroviejo et al.,2003; Su et



al.,2002; Su et al.,2004; Wu et al.,2009] (<http://genome.ucsc.edu/>, genome hg18.) We further investigated *SLC35B4* and sequenced the exons and exon-intron boundaries for all individuals with PHACE syndrome in our cohort, but there were no mutations identified, suggesting that exonic sequence changes in *SLC35B4* were not associated in our cohort of individuals with PHACE syndrome. It is unclear if the haplotype identified for some of the samples could be associated with PHACE syndrome.

The mother of participant #15 has the identical deletion in *AKR1B1* and as far as we know is clinically unaffected. When the mother's CNV regions were compared with child's we identified nine novel deletion/duplication regions in the child's sample. The differences in genetic background, including CNVs inherited by both parents and *de novo* deletions/duplications, could contribute to the presentation of PHACE syndrome via epistatic effects [Eichler et al.,2010]. However, in this case, we feel it is unlikely that this small, inherited deletion in *AKR1B1* is the causative genetic alteration in this patient. Although a single genetic etiology for PHACE syndrome has not been identified, it could be multifactorial and heterogeneous, contributing to the variable clinical presentation. Environmental factors or physical conditions such as pre-eclampsia and premature births may contribute to expression of the phenotype. The clinical variability of PHACE syndrome makes a diagnosis at times tenuous, but important for proper clinical care. So far, PHACE syndrome consists primarily of simplex cases without an obvious inheritance pattern. Although, the deletions observed on 7q33 are not likely the singular cause of PHACE syndrome it is possible that this region provides a genetic susceptibility to phenotypic expression in combination with other inherited or *de novo* CNVs [Girirajan and Eichler,2010; Girirajan et al.,2010].

The presence of segmental hemangiomas is typical for PHACE syndrome. The segmental/unilateral findings in PHACE syndrome provide some support for a two-hit or multistep model. We hypothesize that PHACE syndrome is multifactorial and that there are genetic predisposing factors that in combination with other possible genetic and environmental influences result in the wide phenotypic spectrum of findings seen in PHACE syndrome. Molecular studies on DNA from different tissue types such as the hemangioma tissue looking for somatic events including deletions of the 7q33 region would be interesting. In addition, future studies in larger cohorts investigating germline changes of the 7q33 region will be helpful. It may also be valuable to catalogue the common CNVs between individuals as these may contribute to the variable phenotype observed for PHACE syndrome. Our current cohort provides high resolution copy number change information with 720,000 probes throughout the whole genome in 16 individuals with PHACE syndrome. Although based on only 2 individuals, the 7q33 region is a potential candidate locus for further studies to be undertaken in revealing the etiopathogenesis of this rare syndrome.

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